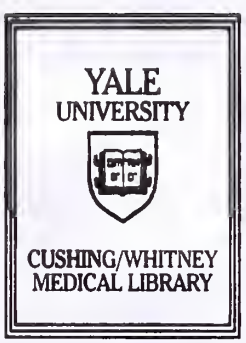
MED
T113
+Y12
7065

CELLULAR DYNAMICS OF KRIT1: IMPLICATIONS FOR THE PATHOGENESIS OF CEREBRAL CAVERNOUS MALFORMATIONS

Nduka M. Amankulor

YALE UNIVERSITY

2004


YALE
UNIVERSITY
CUSHING/WHITNEY
MEDICAL LIBRARY

Permission to photocopy or microfilm processing of this thesis for the purpose of individual scholarly consultation or reference is hereby granted by the author. This permission is not to be interpreted as affecting publication of this work or otherwise placing it in the public domain, and the author reserves all rights of ownership guaranteed under common law protection of unpublished manuscripts.

Signature of Author

05/07/04

Date

Digitized by the Internet Archive
in 2017 with funding from
Arcadia Fund

https://archive.org/details/cellulardynamics00aman

CELLULAR DYNAMICS OF KRIT1: IMPLICATIONS FOR THE PATHOGENESIS OF CEREBRAL CAVERNOUS MALFORMATIONS

A Thesis Submitted to the

Yale University School of Medicine

In Partial Fulfillment of the Requirements for the

Degree of Doctor of Medicine

By

Nduka M. Amankulor

Thesis Advisor: Murat Gunel, M.D.

May 2004

T 113
+Y12
7065

ABSTRACT

A Cerebral cavernous malformation (CCM) is a specific type of vascular malformation that primarily affects the central nervous system and leads to stroke, seizures and focal neurological deficits. Unlike their normal counterparts, vascular channels in characteristic CCM lesions are mere endothelial-cell sinusoids and they lack normal vessel wall components such as smooth muscle, adventitia, and astrocytic foot processes. The absence of these vascular components in CCM lesions suggests a dsyregulation of cellular adhesion and migration mechanisms.

KRIT1 is a gene mutated in a monogenetic, familial form of CCM. Previous work has established the KRIT1 protein as a plus-end microtuble associated protein that also binds integrin cytoplasmic adhesion protein (ICAP-1α). These findings suggest a role for KRIT1 in modulating cell shape and cell adhesion properties. Extending on this body of information, the work in this manuscript suggests that KRIT1 is involved in the control of cell shape dynamics and that KRIT1 over-expression results in gene-expression changes involving a wide variety of cytoskeletal and cell adhesion proteins. Using fluorescence immunocytochemistry we show first that KRIT1 co-localizes with the Rho-GTPase *Rac* at the leading edges of spreading bovine aortic endothelial (BAE) cells. Transfection of full-length KRIT1 into CHO cells results in the formation of cellular processes that are not present in mock transfections or in transfections using only the C-terminal component of KRIT1. Finally, using DNA microarray techniques to compliment the findings made by microscopy, we show that over-expression with a full-length GFP-tagged KRIT1 protein modulates gene-expression in COS7 cells, disproportionately affecting Actin, *Ras*-related proteins and other genes involved in cell shape dynamics and cell adhesion. Taken together, our work suggests novel relationships between KRIT1 and other proteins involved in the formation of new vasculature.

YALE MEDICAL LIBRARY

AUG 2 3 2004

TABLE OF CONTENTS

1. Abstract

2. Acknowledgments

3. Introduction (Pages 1-9)

4. Methods and Materials (Pages 10-14)

5. Results (Pages 15-25)

6. Discussion (Pages 26-30)

7. Conclusion (Page 31)

8. References (Pages 32-40)

ACKNOWLEDGEMENTS

This thesis is dedicated to my mother, who has endured more in her life than any person should have to and whose lessons in strength and perseverance have guided me through my own tough times.

I am incredibly indebted to Murat Gunel, who has given me his unwavering support throughout my efforts in the neurosurgical arena.

I am grateful to Rachel, for her love and support throughout the last three years.

So many people have bolstered my fledgling attempts in science. I am grateful to my labmates, Jennifer Voorhees, Ozlem Kayisli, Katie Pricola, Brian Nahed, Askin Seker and Grahame Gould for their support through the challenging periods of scientific experimentation. I am very grateful to Dr. Richard P. Lifton and to three members of the Lifton Lab, Carol Nelson-Williams, Kris Kahle and Rick Wilson, who dealt daily with my pestering questions.

Michael DiLuna and Maxwell Laurans, my future co-residents, have provided me with much advice over the past few years and for this I owe them a great deal of gratitude.

Finally, I am grateful to my brothers, Chinedu and Chimereze, and to my late father, Professor James N. Amankulor, who, through their love of knowledge, inspired my own.

INTRODUCTION

A History of Central Nervous System Vascular Malformations

Vascular channels of the brain and spinal cord are paramount for normal human function(1). As such, anatomic and physiologic findings related to the cerebral vascular system have been described since the days of Hippocrates(2). These concepts would find new meaning through the work of anatomic giants like Galen and Vesalius(3, 4) and, subsequently, surgical treatment for cerebral vascular diseases would be provided by the founding fathers of neurosurgery. Symptomatic central nervous system vascular diseases were first recognized 2,400 years ago by Hippocrates and termed "apoplexy", a Greek word literally meaning "struck down by violence(2)." Recognizing the diverse pathways leading to stroke or "apoplexy", early 19th century physicians coined the term "sanguineous apoplexy," a term roughly equivalent to our modern day "hemorrhagic stroke(2)." It was not until the mid-1800s that Rudolf Virchow would recognize cerebral vascular malformations as a significant cause of hemorrhagic stroke(2).

Cerebral Vascular Malformations

Our modern conceptions of cerebral vascular malformations are primarily due to the contributions of Cushing, Bailey and Dandy(1). In 1928, Cushing and Bailey described their operative experiences with cerebral vascular malformations (CVMs), a group of vascular lesions that had been previously classified under the misnomer of

"angiomatous tumors." They described three anatomically distinct CVMs: angioma venosum, angioma arteriale and telengiectasias. These descriptions, based largely on histopathological findings, are still largely relevant today.

Although several unique types of CVMs have been discovered, many observers agree that they may be uniformly characterized as cerebral vascular harmatomas resulting from embryologic vascular dysgenesis(5). Generally, CVMs can be distinguished on the basis of a few histopathological findings, namely: a) the type of vascular channel involved (arterial or venous); b) the presence or absence of intervening neural parenchyma; and c) the presence or absence of direct and abnormal connections between arterial and venous vascular systems(5).

Arterial Venous Malformations

Arteriovenous malformations (AVMs) are the most widely recognized of the CVM family, occurring with a prevalence of nearly 1 in 100 people(1, 6, 7). AVMs are distinguished from other CVMs by the presence of direct arterial-venous vascular channels and the concomitant absence of an intervening capillary bed(1). Clinical manifestations of AVMs can be understood by appreciating their physiology. In AVMs, a high pressure arterial system feeds a lower pressure venous system whose vasculature is ill-equipped for the high pressure vascular flow(8). Ultimately, the venous channels compensate by becoming tortuous and dilated. Unfortunately, these dilated, tortuous vessels are prone to enlargement and hemorrhage, leading to the clinical symptoms associated with AVMs(8). Rarely, the propensity for AVMs can be inherited(9). Hereditary hemorrhagic telengiectasias (HHT), or Osler-Weber-Rendu, affects 1 in

100,000 people and can potentially result in AVMs of the brain, nasal mucosa, lung or gastrointestinal system(10, 11). In addition, HHT patients can present with capillary telengiectasias (see below) of any organ. Wyburn-Mason syndrome also known as Bonnet-Dechaume-Blanc syndrome is another rare familial form of AVM in which lesions typically affect the mesencephalon in addition to the retina(12).

Cerebral Venous Malformations

Cerebral venous malformations (VMs), also known as "venous angiomas," or "venous caput medusae," are characterized by grossly enlarged vessels of venous origin separated by normal neural parenchymal tissue(1). Although the incidence of VMs in post-mortem studies nears 3%, they are low-pressure systems which rarely result in hemorrhage and are consequently rarely diagnosed(1). Unlike their counterparts, these malformations are functional and provide normal drainage to surrounding neural tissue. Non-neural venous malformations can be inherited as part of a syndrome known as Venous Malformations, Cutaneous and Mucosal (VMCM1), which result in venous malformations affecting the spleen, liver, pancreas and other organs(1).

Capillary Telengiectasias

Capillary telengiectasias (CTs) are thought to arise from a failure of fetal cerebral capillary involution and are inherited at a rate of 2 per 100,000(1). They appear as dilated end-capillaries with normal intervening neural parenchyma. CTs are often found in HHT syndromes, commonly arising within the pons(1).

Cerebral Cavernous Malformations

Cerebral cavernous malformations are dilated sinusoidal channels without intervening neural parenchyma(1). They have been variously described as "cavernous

hemangiomas," "cavernomas" and "cavernous malformations." Magnetic resonance imaging (MRI) autopsy series report a post-mortem prevalence of 0.4-0.5%, although the actual rates may be significantly higher(1, 13). A familial form of CCM occurs with greater frequency among Hispanics, but this disease can frequently exist among other ethnic groups(14). Lesions can be located anywhere in the brain and spinal cord with equal probability, but they are most frequently found within the pons and the subcortical white matter because of the relatively large tissue densities associated with these structures(1).

Histopathologically, the salient features of CCMs are dilated, tortuous, vascular sinusoids composed of a single layer of endothelial cells (Figure 1)(15). Notably absent from these structures are smooth muscle, adventitia, astrocytic foot processes and other components of normal vessel walls(16). Grossly, these lesions are dark,, multi-lobulated and often described as having a "mulberry-like" appearance(1). The luminal pressure in these CCMs is usually somewhere between those of arterial and venous pressures, and may be dependent on the location of the lesions among other factors(1).

Treatment of Cerebral Vascular Malformations

The treatment heuristics for CVMs can be quite complicated(17). Prior to treating these lesions, one must first consider their natural history. While AVMs have a moderate risk of hemorrhage and usually require treatment, clinically silent venous malformations often simply require expectant management. Various other factors such as patient symptoms, lesion location and size, lesion location and other considerations must be evaluated. Once thorough information has been gleaned, expectant management,

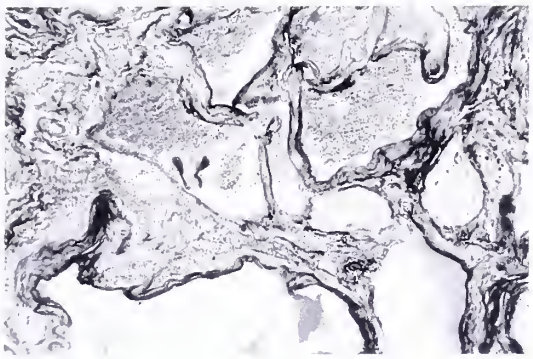


Figure 1: Histological features of a Cerebral Cavernous Malformation

A typical cerebral cavernous malformation showing dilated vascular channels with no intervening neural parenchyma.

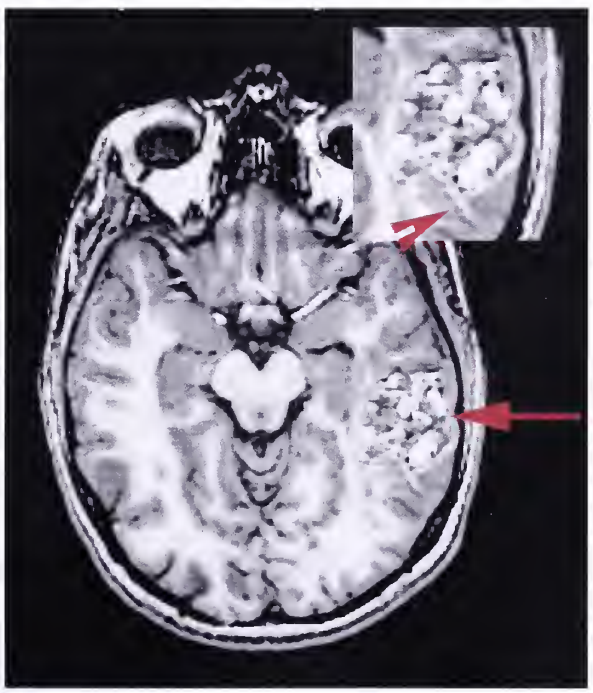


Figure 2: T1-weighted MRI image revealing a cavernous malformation.

Cavernous malformations are characterized by a lesion of mixed signal intensity (inset, arrows), consistent with ongoing hemorrhage. Vascular channels, represented by flow voids, are also present.

endovascular embolization, microsurgical resection and stereotactic radiosurgery are all valid options for treatment(6, 17).

Molecular Features of Cerebral Vascular Malformations

Molecular Genetics of Cerebral Vascular Malformations

Early investigations into the nature of cerebral vascular malformations yielded three factors that suggested a genetic component for CVMs: a) some individuals display multiple CVM lesion that appear early in life; b) certain CVMs were demonstrated to be inheritable and c) different types of CVMs can occur simultaneously in the same person, suggesting a genetic basis(14). With the advent of polymerase chain reaction (PCR) and positional cloning techniques, the molecular genetic features of CVMs are being elucidated(18).

Remarkably, autosomal dominant germline mutations have been associated with the inheritance of all four vascular malformations discussed here (AVMs, CCMs, VMs and CTs). HHT, in which patients display both AVMs and CTs, is associated with two possible genetic loci (HHT1, HHT2). HHT1, located on chromosome 9q34.1(19-21), encodes a protein called endoglin (ENG)(22, 23), while HHT2, located on chromosome 12q13, encodes a protein known as activin receptor-like kinase 1 (ALK1)(24-26). Venous malformations inherited in an autosomal dominant fashion are caused by a mutation in chromosome 9p21, in a region encoding for the endothelial cell promoter TIE2(27, 28). Finally, cavernous malformations may be caused by mutations on chromosome 7q21 (CCM1), 7p13 (CCM2), or 3q25-27 (CCM3)(29-31). While CCM1

and CCM2 encode the proteins KRIT1 and Malcavernin, respectively, the gene at the CCM3 locus is currently unknown(32, 33).

Molecular and Cellular Biology of Cerebral Cavernous Malformations

Cerebral vascular malformations are endothelial cell phenotypes, and they likely represent aberrations in endothelial cell signaling. With the exception of the newly discovered Malcavernin gene associated with the CCM2 locus, the function of each gene associated with CVMs has been definitively linked to endothelial cells(34-36). The expression of KRIT1 protein, the protein of interest for this thesis, has been linked to endothelial cells immunocytochemistry and immunohistochemistry experiments(35). Beyond these particulars, little is known about the molecular pathogenesis underlying the formation of cavernous malformations.

The KRIT1 protein was initially discovered through a yeast two-hybrid screen looking for binding partners for *Krev1* (*Rap1A*)(37). KRIT1 consequently stands for **K**rev1 **R**ap1A **I**nteraction **T**rapped 1 (KRIT1). *Rap1A* is GTPase with sequence homology to the *Ras* oncogene family(38). In spite of this initial connection between *Ras* homologues and KRIT1, there has been little biological proof linking KRIT1, or CCM formation, to a pathogenic mechanism involving *Ras* homologues.

Attempts to confirm a phy(39)sical KRIT1-*Rap1A* interaction using KRIT1 as a yeast two-hybrid bait have yielded somewhat ambiguous results(40, 41). However, these same experiments definitively identified integrin cytoplasmic domain-associated protein 1-α (ICAP 1α) as a KRIT1 binding partner. ICAP 1α is a β–integrin binding protein which apparently modulates cellular adhesion upon integrin engagement(39). While the

consequences of ICAP 1α–integrin binding remain to be fully elucidated, some molecular properties of ICAP 1α are known. For example, it is known that ICAP 1α binds β–integrin through its protein tyrosine binding (PTB) domain, a domain that may have general significance in the pathogenesis of cavernous malformations(33). Since integrins recruit Ras homologues to their downstream signaling mechanisms, the discovery of ICAP 1α potentially bridges the gap between KRIT1 involvement in vascular development and its relationship with Ras signaling(42).

Attempts at elucidating the exact mechanism of KRIT1 action have yielded two interesting findings. First, our laboratory showed that KRIT1 is a plus-end microtubule-binding in bovine aortic endothelial (BAE) cells(34). Secondly, through immunocytochemistry experiments, we have also shown that KRIT1-specific antibodies stain arterial microvascular structures more readily than venous structures or large arterial structures(36). These two findings set the stage for further investigations into the pathogenesis of cavernous malformations. Specifically, they suggest a role for KRIT1 in angiogenesis, perhaps by control of the cellular cytoskeleton.

Recently, Marchuk and colleagues identified the gene responsible for cavernous malformations at the CCM2 locus, leaving CCM3 as the only unidentified gene(33). The CCM2 gene encodes a protein named Malcavernin, which, like ICAP 1α, contains a putative protein tyrosine binding (PTB) domain(33). Although its biological properties are currently unknown, the presence of PTB domain within Malcavernin suggests that this protein may also be involved in integrin signaling(39).

Rationale for Investigations into the Function of the KRIT1 Protein

Cavernous malformations are an important cause of hemorrhagic stroke. Investigations into CCM lesions with Mendelian patterns of transmission provide an opportunity to study single genes which may be generally important in the etiology of strokes. By understanding the molecular and cellular properties of the KRIT1 protein, we might gain better insight into the pathogenesis of hemorrhagic strokes.

Since 1999, it has been known that mutations in the KRIT1 gene lead to cavernous malformations(32). Yet, besides the discovery that the KRIT1 protein is a microtubule-associated protein that also binds ICAP 1α , little else is known about the function of this protein. This dearth of information on the KRIT1 protein has forced us to seek new ways of examining the KRIT1 protein.

The presence of multiple peptide domains within KRIT1 raises the possibility that each domain may be studied individually to understand how the domains affect the overall function of the KRIT1 protein. We hypothesized that over-expression of KRIT1 in cells which do not usually express KRIT1 would lead to the observation of KRIT1-specific cellular phenotypes. Finally, through the use of DNA microarrary as a tool to study gene expression changes in cells over-expressing KRIT1, we hypothesized the identification of new genes that may be related to KRIT1 function and CCM pathogenesis.

METHODS AND MATERIALS

(DNA microarray hybridization experiments were performed with the assistance of Irina Tikhanova and Grahame Gould. All other experiments were performed by the author.)

Preparation of KRIT1-GFP Constructs. We generated a series of KRIT1 over-expression constructs fused to the N-terminus of the green fluorescent protein (GFP) coding sequence in an EGFP-N3 vector (BD Clontech) for over-expression and localization experiments. KRT1 was amplified from mouse brain and human kidney DNA libraries (Richard Lifton's laboratory, Yale School of Medicine). A full-length (FL) KRIT1 was amplified by a using a (sense – 5' AGCGCAATCTCGAGAAGCCACCATGGGAAATCCAGAAAACATG 3' antisense 5' AGCGCAAATAAGCTTATGAATTTCTTTCAGAGGGCAT 3') primer set. An N-terminus KRIT1 amplicon was made using a (sense 5' AGCGCAATCTCGAGAAGCCACCATGGGAAATCCAGAAAACATG 3' and antisense 5' AGCGCAAATAAGCTTAGCTCATGCTTCGCTGCCATTT 3') primer set. The C-terminus KRIT1 amplicon was made using a (sense 5' AGCGCAATCTCGAGAAGCCACCATGAAATGGCAGCGAAGCATGAGC 3' and antisense 5' AGCGCAAATAAGCTTATGAATTTCTTTCAGAGGGCAT 3') primer set. The Hispanic mutant KRIT1 sequence which containing a 2105 C→T mutation was constructed using a (sense – 5' AGCGCAATCTCGAGAAGCCACCATGGGAAATCCAGAAAACATG 3' and antisense 5' AGCGCAATAAGCTTGTTGAGAGAGACGCATTCCTTCCA 3') primer

set. All primers contained internal Kozak consensus sequence (5' ACCATGG 3') which was incorporated into the sequence of all PCR amplicons. Resultant amplicons and the E-GFP N3 vector were digested with restriction enzymes Xho I (New England Biolabs, Beverly MA) at the 5' position and HindIII (New England Biolabs, Beverly MA) at the 3' position. Reactions were carried out at 37° C for 1 hour. Constructs were ligated into the E-GFP N3 vector using DNA Ligase (New England Biolabs, Beverly MA) at 4° C overnight. The ligated mixture was grown in Super Competent E. Coli cells and positive colonies were picked. Colonies were grown in LB medium and mini-preps performed with a Mini-kit (Qiagen,Valencia CA). DNA obtained from Qiagen kits were sequenced (Keck Sequencing Laboratory, Howard Hughes Medical Institute, Yale Medical School) and verified using various a GFP (sense 5' GCTAGCGCTACCGGACTCAGATCT 3' and various KRIT antisense) primer sets.

In vitro cell culture. COS7 cells (ATCC, Rockville MD) were generally grown 10cm culture dishes with low glucose 10ml Dulbecco's Modified Eagle Medium (DMEM) (GIBCO BRL, Carlsbad CA) supplemented with 10% Fetal Bovine Serum (GIBCO), 10mM sodium pyruvate, 10mM HEPES buffer, 100 U mL^{-1} penicillin, 100 mg mL^{-1} streptomycin and 0.25 mg mL^{-1} amphotericin B. CHOK1 and BAEC cells were grown in 10ml high glucose Dulbecco's Modified Eagle Medium (DMEM) (GIBCO BRL, Carlsbad CA) supplemented with 10% Fetal Bovine Serum (GIBCO), 10mM sodium pyruvate, 10mM HEPES buffer, 100 U mL^{-1} penicillin, 100 mg mL^{-1} streptomycin and 0.25 mg mL^{-1} amphotericin. Cell culture was maintained by washing cells with PBS (Sigma, Saint Louis MO) and treating with 1mL

Immunocytochemistry. Cells were grown on microscope slides and allowed to grow to 50-70% confluence. On the day of an experiment, cells were washed with PBS (Sigma) and coverslips were removed from Petri dishes and placed into a humid chamber. Cells were fixed with 3-5% paraformaldehyde, permeabilized using 0.2% Triton-x100 and blocked with 10% Normal Donkey Serum (Jackson ImmunoLaboratories, West Grove PA) and and 1% Bovine Serum Albumin (BSA) in PBS. Cells were incubated with primary antibodies using Rac-1 mouse monoclonal (Upstate, Waltham MA), anti-vinculin monoclonal (Sigma, Saint Louis MO) or rabbit polyclonal KRIT1 antibodies (see Laurans, MSH for characterization). Cells were then incubated with donkey FITC-anti rabbit or Cy3 donkey anti-mouse secondary antibodies (Jackson ImmunoLaboratories, West Grove PA), washed several times with PBS and then stained with DAPI for nuclear staining. For immunocytochemistry experiments with KRIT-GFP transfected cells, cells were simply washed with PBS, fixed with paraformaldehyde as above and mounted unto slides for fluorescence microscopy. To characterize the length of cellular processes, cells were viewed at 40X magnification under the live camera microscope (Zeiss). Processes extending from the cell body were measured on the screen using a ruler. Counting of cells was begun in the upper left quadrant for slide and viewing was conducted in a counter-clockwise direction to insure that cells were not duplicated in the count.

Hybridization of DNA microarray slides. These experiments were performed with the help of Irina Tikhanova and Grahame Gould. Microarray experiments reported here were all carried out using a 900™ kit from Genisphere (Hatfield PA). Total cellular RNA was obtained from KRIT-GFP transfected cells using an RNeasy kit (Qiagen, Valencia CA)

and characterized for purity by spectrophotometry. Total cellular cDNA was obtained by RT-PCR using superscriptII reverse transcriptase (Invitrogen, Carlsbad CA). To reduce backround on the micorarray plate, microarrays were pre-hybridized by applying a pre-hybridization mix heated to 80° C and incubated for 1-2 hours at 50° C.

Analysis of microarray data. A scanned image of our microarray was imported into the GenePix 4.0 folder and loaded unto an appropriate 21,000 gene grid. The gene grid was then manually manipulated to fit the microarray image and genes which did not hybridize well (i.e. absent signals or uneven signals) were marked and thus considered ineligible for analysis. After formatting the grid as described above, an initial round of analysis was performed, giving us several thousand discrete data points to consider. To measure the intensity of the hybridization in a given channel (Cy3 or Cy5), we used the mean value observed for a given fluor at a single gene as the point of analysis. Only genes whose mean values were 2X greater than the mean value of all genes included in the initial analysis were considered valid (Figure . The degree of over-expression or under-expression was assessed by considering the median value of the ratio of Cy5/Cy3 channels. Only genes with a median of ratios two standard deviations greater than the average median of ratios were considered as over-expressed. Under-expression was set at the same corresponding value, except in the negative direction.

RT-PCR of KRIT1-GFP RNA. Total cellular RNA from KRIT1-GFP transfected cells was obtained using an RNeasy kit (Qiagen, Valencia CA). Total cellular DNA was obtained using reverse transcription with Superscript reverse transcriptase (XXX). C-terminal KRIT1-GFP, full-length KRIT1-GFP and the Hispanic mutant KRIT1-GFP PCR amplicons were obtained using a (sense – 5' TAGCACAAGGCATATTGGAC 3' and

antisense 5' GTCTTCAGTTCAAGCCTATC 3') primer set yielding a 912 bp fragment. DNA from N-terminus KRIT1 GFP was amplified using (sense – 5' GTCCAATATGCCTTGTGCTA 3' and antisense GTCCAATATGCCTTGTGCTA) primers yielding a 390 bp fragment. PCR products were run on 0.8% agarose gels and visualized by addition of Ethidium Bromide.

Transfection of KRIT-GFP Constructs into COS7 and CHOK1 cell lines. Cells were grown to 70% confluence in 10cm cell culture dishes. On the day of transfection, cells were washed with 1X PBS (Sigma, Saint Louis, MO) and placed in 12ml of Optimem™ media (GIBCO) 15 minutes prior to transfection. Transfections were carried out in 10cm culture dishes with coverslips placed at the bottom of the dish for future microscopy experiments. For each transfection, 15μg of DNA was placed in 1ml of Optimem and incubated for 10 minutes. At the same time, 80μl of Lipofectamine 2000 reagent (Invitrogen, Carlsbad CA) was placed in a separate vessel and incubated with 1ml of Optimem for 10 minutes. The two reactions were mixed, gently tapped, and then incubated for 20 minutes prior to addition into a 10cm dish. 4 hours after addition of the DNA/Lipofectamine mixture, the media was removed from culture dishes. Cultures dishes were washed once with PBS and the normal cell media was replaced. Cells were assessed by fluorescent microscopy at 12, 24, 36 and 48 hours for maximum GFP expression.

RESULTS

KRIT1-GFP constructs are expressed in COS7 cells. COS7 cellular expression of the various KRIT1-GFP constructs was verified by RT-PCR (Figure 6b) or Western blot analysis (Figure 6a). COS7 cells transfected with KRIT1-GFP constructs express KRIT1 RNA fragments (Figures 6a), whereas COS7 cells transfected with an empty GFP vector expresses no such fragment. In addition, cells transfected with a C-terminus KRIT1-GFP construct or an N-terminus KRIT1-GFP construct yield anti-GFP antibody bands of the expected sizes (Figure 6b). Anti-GFP western blots of cells transfected with an empty GFP vector also results in a protein of the expected size (25KDa).

KRIT1 co-localizes with Rac1 in BAEC cells grown on fibronectin. Based on prior evidence showing KRIT1 interaction with integrin cytoplasmic adhesion protein 1 α (ICAP-1α,), we wondered if KRIT1 localization was affected by other integrin-dependent cellular events. To investigate this hypothesis, BAEC cells coated with fibronectin (an integrin-receptor substrate) were grown for 72h and immunocytochemistry using KRIT1 antibodies was performed. Fibronectin coating resulted in increased cell spreading for all cells viewed. Immunocytochemistry of BAEC cells coated with fibronectin showed KRIT1 localization to membrane ruffles and the leading edge of the BAEC cell membrane (Figure 3), a pattern of staining which differed from our previously observed microtubular staining in non-spreading cells. To further characterize these observations, double staining of KRIT1 and Rac1 (a protein known to localize to membrane ruffles in an integrin-dependent manner was perfomed.. Figure 3 shows co-localization of Rac1 and KRIT1 in BAEC cells coated with fibronectin. In

order to observe the extent of KRIT1 involvement in integrin-dependent cellular processes, we wondered if KRIT1 also localized to integrin-dependent focal adhesions stained by vinculin. Our results show that while KRIT1 is associated with Rac1 at membrane ruffles and the leading edge of BAEC cells, there is no co-localization of KRIT1 in focal adhesions (Figure 4). These results suggest that KRIT1 may be involved in some, but not all integrin-dependent changes resulting cellular and cytoskeletal morphology.

Transfection of KRIT1-GFP induces changes in cellular morphology. COS7 cells transfected with various KRIT1-GFP constructs were grown for 36-48 hours, when cells displayed maximal expression of green fluorescent protein (GFP). Cells were then fixed and observed under fluorescent microscopy at 40X magnification (Zeiss fluorescence microscope). Our results indicate that transfection of KRIT1-GFP constructs into COS7 cells results in distinctive changes to cellular morphology (Figure 8). Particularly, the presence of the N-terminus KRIT1-GFP induces long, thin cellular processes (Figure 8, NT, FL), , whereas transfection GFP vector causes no observed changes to cellular morphology (Figure 8, CT), When we measure the number of cellular processes greater than 3cm in length (at 40X magnification), we see greater than a 5-fold increase in these cell processes when compared to normal GFP controls (Figure 9). Transfection of C-terminal KRIT1-GFP resulted what qualitatively observed large cells (Figure 8, CT); however, we have not quantified the degree of CT KRIT1-GFP induced cell spreading. Overall results suggest that an unknown KRIT1 N-terminus sequence is responsible for

causing extensive changes in cellular morphology marked by induction of long, thin cellular processes.

DNA microarray experiments show differential expression of KRIT1-GFP transfected cell. After observing changes in the cellular morphology of KRIT1 transfected COS7 cells, we were eager to determine if differential expression profiles of certain genes could be responsible for these observed morphologies. As an initial attempt to determine differentially expressed genes in KRIT1 transfected cells, DNA microarray hybridization experiments were performed on C-terminal KRIT1-GFP and full length KRIT1-GFP transfected cells (Figure 7, Table1, Table 2). First, our results show that transfection of C-terminal KRIT1-GFP induces up-regulation of actin homologues and actin-binding proteins suggesting active cytoskeletal restructuring by the KRIT1 C-terminus (Table 1). Notably, C-terminal KRIT1-GFP produced more up-regulated than down-regulated genes (Figure 10). Transfection of full-length KRIT1-GFP into COS7 cells induces a distinct modulatory pattern on gene expression where many more genes are down regulated than up regulated (Figure 10). Interestingly, full-length KRIT1 induced the up-regulation of two Ras-related genes and another gene (Thrombospondin 4) that is known to mediate neurite outgrowth (Table 2). Many of the genes suppressed by full length are pro-apoptotic genes, leading to the conjecture that full-length KRIT1 may be acting as an anti-apoptotic element, an observation made before in our laboratory (O. Kayisli, personal correspondence)

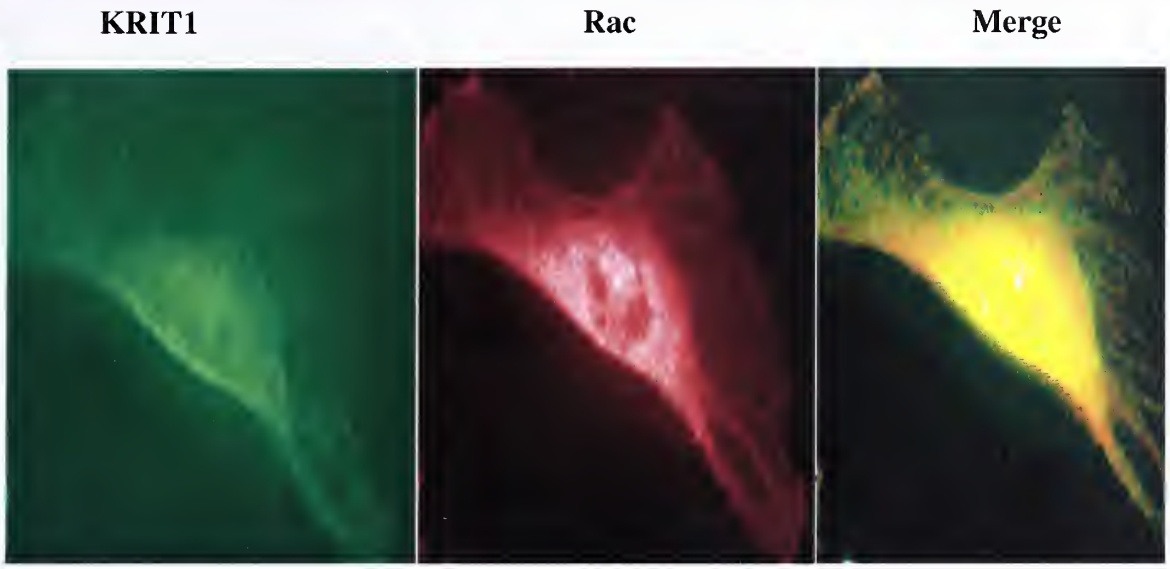


Figure 3: KRIT1 and Rac co-localization in spreading BAEC cells

BAEC cells were plated at 20% confluence on fibronectin and allowed to spread for 72 hours. Immunocytochemistry of cells BAEC cells showing KRIT1 (green) and Rac1 (red) co-localization to membrane edges and membrane ruffles.

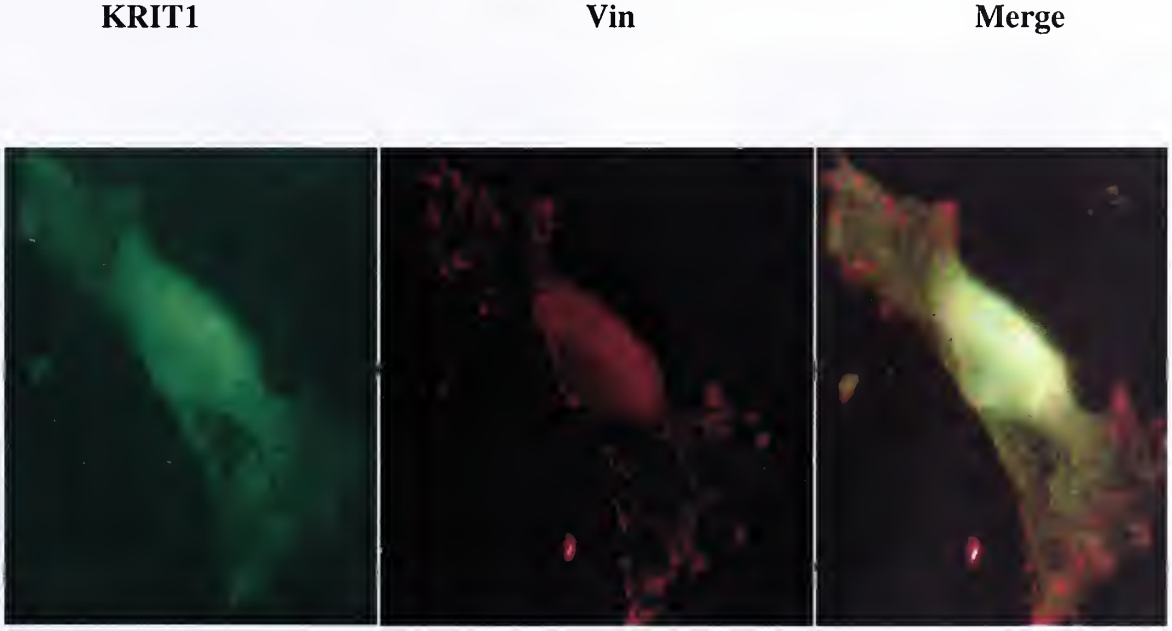


Figure 4: KRIT1 localizes to membrane edges, but not focal adhesions

Spreading BAEC cells, as in Figure X were double-stained with KRIT1 and vinculin (vin) to assess whether KRIT1 localizes to focal adhesions (stained by vinculin). Our results show that KRIT1 (green) and vinculin (red) co-localize to distinct compartments of the membrane edge

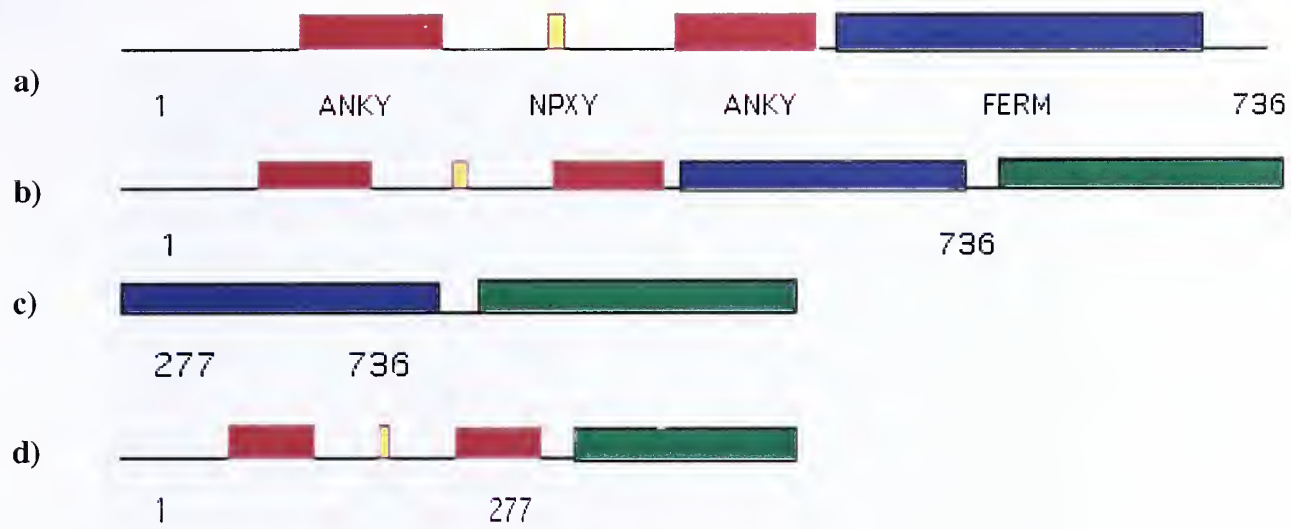


Figure 5: Schematic representation of KRIT1-GFP constructs.

Normal KRIT1 (a), full-length KRIT1-GFP (b), KRIT-1 C-terminus-GFP (c), and N-terminus-KRIT1-GFP constructs (d) are shown. Numbers represent amino acid sequences of KRIT1 (1-736 amino acids). Red=ankyrin domain; orange=NPXY domain; dark blue=FERM domain; green=GFP domain.

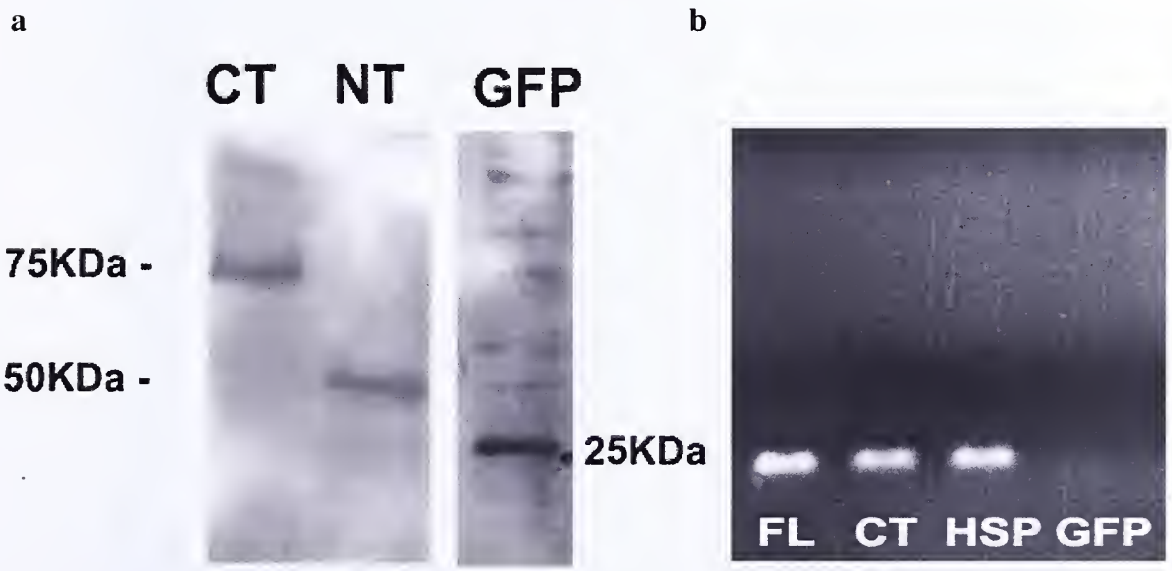


Figure 6: Western blot and RT-PCR of KRIT1 transfected Cos 7 cells.

Lysates from Cos7 cells transfected with C-terminus KRIT1 (CT), N-terminus KRIT1 (NT) and a GFP vector (GFP) were obtained and immunoprecipitated with a monoclonal anti-GFP antibody. Results from representative experiments show the expected ~75KDa, ~50KDa and ~25KDa bands that are expected for CT, NT and GFP, respectively. Full-length KRIT1 (FL) Western blot (not shown) showed a band of the expected size. RT-PCR of lysates from the same transfection showing Cos7 expression of (FL, CT, HSP=Hispanic mutant). KRIT1 expression is absent in GFP tranfected cells.

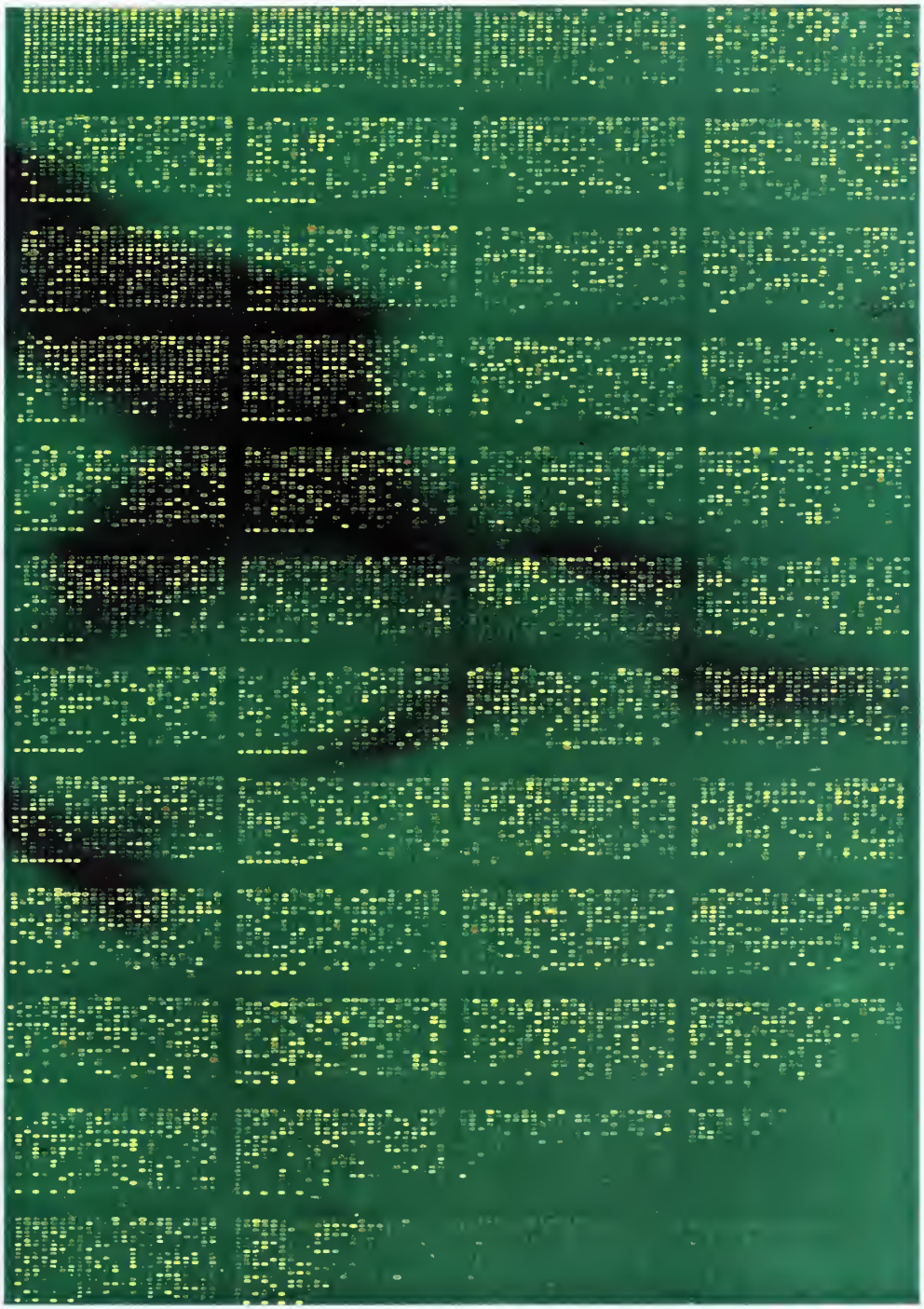
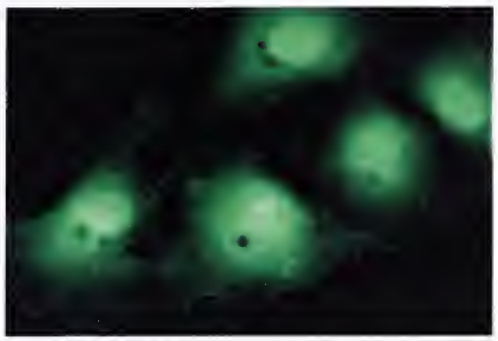
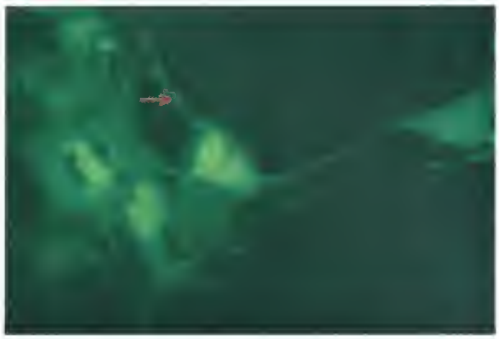


Figure 7: Hybridization of DNA from KRIT1-transfected cells to microarray panel

DNA microarray panel shows adequate hybridization of DNA from KRIT1-transfected cells. All parts of the panel are hybridized except for lower left part of the plate (excluded from diagnosis). Similar images were obtained for KRIT1 C-terminus transfected cells.

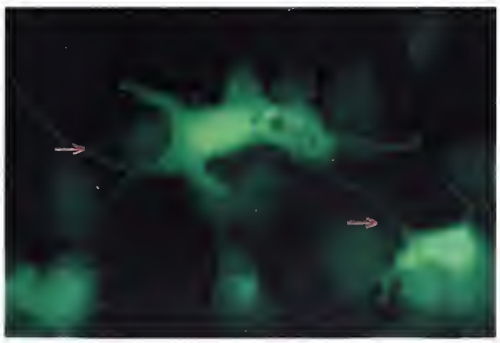


GFP



FL

CT



NT

Figure 8: Fluorescence microscopy images from GFP or KRT1-transfected Cos 7 cells (40X magnification)

COS7 cells were transfected with constructs expressing an empty GFP vector or various KRIT1-GFP fusion constructs. Cells were fixed and viewed under a fluorescence microscope 48 hours after transfection. A) Representative cells from transfection with an empty GFP vector resulted in non-specific cell spreading. B) A similar phenotype was found with transfection of the C-terminus KRIT1-GFP template. C-terminus KRIT1-GFP transfected cells were qualitatively found to be much broader than all other cells. Transfection of the N-terminus of KRIT1, either by using N-terminus KRIT1-GFP (NT) or full-length KRIT1-GFPconstructs (FL) resulted in the formation of extensive cellular process which often made contact with surrounding cells (red arrows).

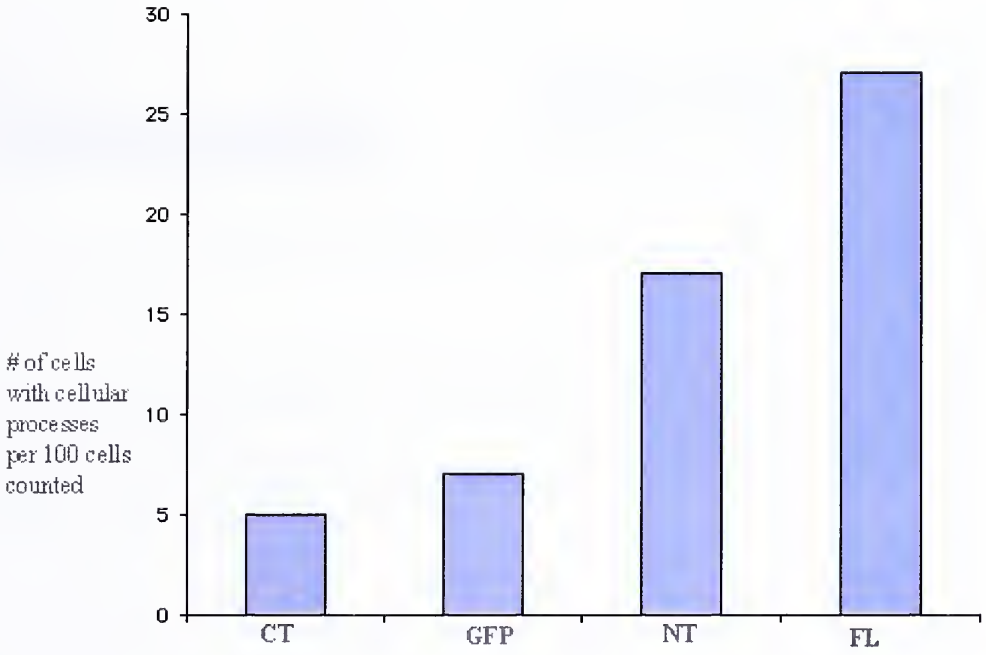


Figure 9: Assessment of cellular processes in cells transfected with KRT1 constructs

Cellular processes were identified in KRIT1-GFP transfected cells and those processes greater than 3 centimeters at 40X magnification (Figure 8) were included in our analysis. Full-length KRIT1-GFP (FL), N-terminal KRIT1 GFP (NT), the empty green fluorescent protein (GFP) vector and C-terminal KRIT1-GFP (CT) are shown.

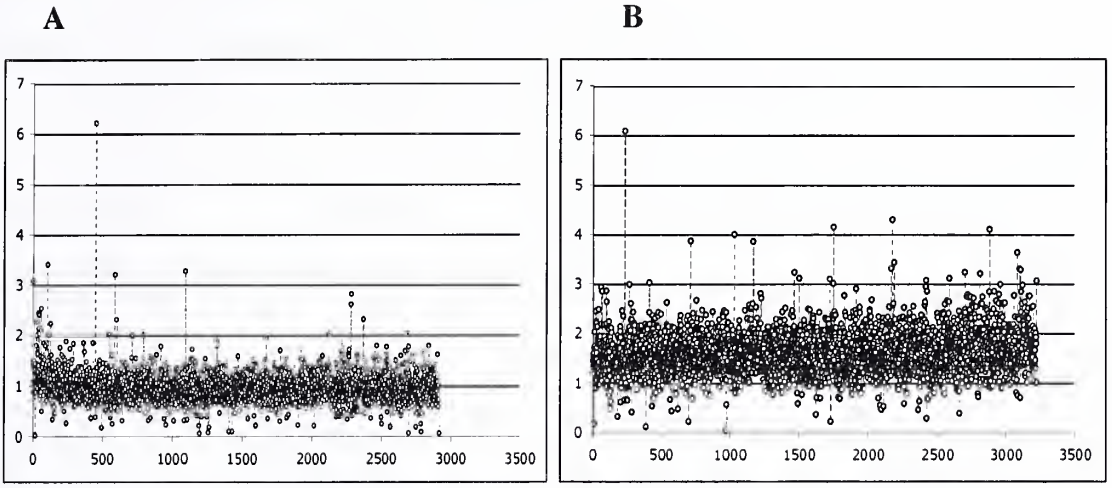


Figure 9: Scatter plot of genes with adequate hybridization following microarray with KRIT1-GFP transfects

Scatter plots representing the total number of genes considered for analysis in the KRIT1-GFP overexpression experiments. For C-terminal (CT) experiments, 3,217 discrete genes met the criteria for consideration. (Mean median of ratios= .975; StdDev=0.78). Overexpression with full-length (FL) fragments provided 2,918 valid genes for analysis (Mean median of ratios=.831; StdDev=0.82).

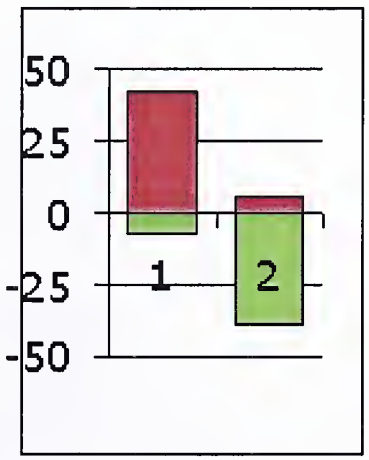


Figure 10: Total number of genes with significantly modulated expression following KRIT1-GFP overexpression.

For C-terminal KRIT1-GFP (1), 42 genes were up-regulated while 7 were down-regulated; conversely, full-length microarray experiments (2) produced only 6 up-regulated genes and 39 down-regulated genes.

Table 1

Modulation of Gene Expression after C-terminal KRIT1-GFP transfection of COS7 cells

Gene/Protein	Accession No.	Degree of change	Comments
Cell shape/ cycle			
Keratin 7	NM 005556	+2.636	Intermediate filament
Ezrin	NM 003379	+2.601	FERM domain family
Tubulin beta 5	NM 032525	+2.565	Tubulin cytoskeleton
FLJ23324 fis	AK026977	+2.521	Myosin 10
Actinin alpha 1	NM 001102	+2.498	Focal adhesions
Actin gamma 1	NM 001614	+2.452	Actin family
FLJ32120 fis	AK056682	+2.439	Actin beta-like
Cofilin 1	NM 005507	+2.424	Cell differentiation
LMHX6	NM 014368	+2.416	Actin-binding
PRO1855	NM 018509	+2.404	Flightless homolog
Transgelin 2	NM 003564	+2.37	Actin-binding
DKFZp43	AL133645	+2.36	Actin-binding
Actin beta	NM 001101	+2.354	Actin protein
Adhesion/Matrix			
SLITRK3	NM 014926	+2.848	Slit gene homologue
COL9A2	NM 001852	+2.638	Fibrillar collagen
CDH17	NM 004063	+2.565	Cadherin family
VGF inducible	NM 003378	+2.487	Nerve growth factor
ADAMTS2	NM 014244	-0.244	Metalloproteinase
Endocytosis			
TOMM40 homolog	NM 006114	+2.743	Mitochondrial
EPN1	NM 013333	+2.593	Clathrin vesicles
LAPTMB4	AY057051	+2.432	Lysososmes
BIN1	NM 004305	+2.414	Clathrin vesicles

Table 2

Modulation of Gene Expression after Full length KRIT1-GFP transfection of COS7 cells

Gene/Protein	Accession No.	Degree of change	Comments
Cell shape/angiogenesis			
RHEB	NM 005614	+6.209	Ras GTPase
ADM	NM 001124	+3.274	↑ angionesis
THBS4	NM 003248	+2.614	↑ neurites
DFKZp434D152	AL136835	+2.54	Ras GAP
*TIMP3**	NM 000362	-3.8	↑ angionesis
*MGC11256**	NM 024324	-9.43	Fibrillin-like
LMNB1	NM 005573	-9.52	Nuc. filament
Apoptosis			
PPP1R15A	NM 014330	-2.52	↑ apoptosis
CDKN1A (p21)	NM 000389	-2.73	↑ G1 arrest
C20orf19	NM 021158	-2.79	↑ apoptosis
HYOU6	NM 006389	-2.85	↑ in hypoxia
DDIT3 (GADD153)	NM 004083	-2.78	↑ apoptosis
*TIMP3**	NM 000362	-3.81	G1 regulation
NAG-1	NM 004684	-5.07	? apoptosis
Adhesion/matrix			
*MGC11256**	NM 024324	-9.43	TG

DISCUSSION

The function of KRIT1 and the exact nature of biological pathways perturbed in cavernous malformations remain to be fully elucidated. One conjecture about the function of KRIT1, based on the work of several investigators, is that KRIT1 may serve as a transducer between β-integrin and the cellular cytoskeleton(43). In this model, β-integrin engagement with an extracellular matrix substrate (such as fibronectin) would lead to appropriate changes to the cellular cytoskeleton. KRIT1 may serve as a direct adaptor between the cytoplasmic face of β–integrin and microtubules. Alternatively, the KRIT1 protein may respond to integrin-engagement by binding ICAP 1α, a protein known to bind β-integrin directly. Whatever the mechanism, these findings provide no definitive proof of KRIT1's role in vascular development.

A recent experiment where investigators generated KRIT1 knockout mice shows that homozygous mutants are embryonic lethal and develop a wide variety of vascular malformations primarily restricted to the arterial vasculature(35). In addition, these mice also show down-regulation of Notch gene expression(35). While this finding establishes cavernous malformations as a pure vascular phenotype, the link between these phenotypes and their underlying molecular features are currently missing.

The work in this thesis provides preliminary information on a number of genes which may be involved in KRIT1 signaling. Through immunocytochemistry experiments on spreading endothelial cells we first show that KRIT1 staining is consistent with the staining of Rac1, a small Ras-related Rho GTPase which requires integrin engagement to translocate to the plasma membrane. From the plasma membrane, Rac1 recruits a number of downstream effectors and has the net effect of modulating the actin

cytoskeleton. Although the relationship between KRIT1 and Rac1 has not been fully explored, it is intriguing to suggest that the KRIT1 protein may be part of a coordinated response to integrin engagement. Notably we also show here that, KRIT1, like Rac1, is not present in integrin-dependent focal adhesions(44). This finding may mean that KRIT1 is involved in some specific integrin-based signals but not others. One intriguing possibility is that this specificity is conferred by the type of extracellular matrix protein which binds the integrin receptor.

The coordinated actions of integrins, Rho-GTPases such as Rac and the actin cytoskeleton lead to changes in cell shape and to the exploration of the cellular micro-environment through the formation of actin-based cellular processes known as filopodia and lamellipodia(45). It is therefore interesting that over-expression of full-length KRIT1 constructs in COS7 cells leads to an increase in the number of cellular processes. While the nature of these cellular processes are currently unknown, it would be worthwhile to see if they involve active actin remodeling and if Rho-GTPases are required for their formation.

Perhaps the most intriguing of the findings made here are the genes which are modulated by over-expression of KRIT1-GFP constructs in our microarray experiments. Although these results are preliminary, the nature of the modulated genes deserves some discussion. Over-expression of full-length KRIT1 results in the up-regulation of a number of interesting genes. One such gene, Ras homologue enriched in brain (RHEB) is a Ras homologue which has been implicated in cell growth(46-48). Interestingly, RHEB is involved in regulation the TSC1/TSC2 tumor-suppresor complex, a complex of genes mutated in tuberous sclerosis(49). Another Ras-related protein, *DFKZp434D152*,

is a putative Ras GTPase activating protein (RAS-GAP); however, its physiologic function is less clear than RHEB. Adrenomedullin (ADM), a gene up-regulated by full-length KRIT1 expression has been shown to promote vascular proliferation and migration and signals through the phosphatidylinositol 3-kinase (PI3K) and Akt(50, 51). Another interesting gene up-regulated by full-length KRIT1 over-expression is Thrombospondin 4, a gene which has been shown to increase neurite outgrowth(52). This finding is particularly interesting in the context of full-length KRIT1 induction of cellular processes.

Microarray experiments performed after C-terminal KRIT1 over-expression in COS7 cells also reveal some very interesting genes. The C-terminus of KRIT1 includes a Band 4.1, Ezrin, Radixin, Moesin (FERM) domain(53). This domain is included in several important proteins including the Neurofibromatosis 2 (Nf2) gene product, Merlin(54). FERM domain-containing proteins are hypothesized to tether actin microfilaments to the plasma membrane(53). This mechanism provides yet another way for the cellular cytoskeleton to respond to changes at the cellular plasma membrane. Given this context, the genes modulated by C-terminal KRIT1 (CT) over-expression appear consistent with the proposed mechanism of KRIT1 action. Ezrin, one of the founding members of the FERM domain family is significantly up-regulated after CT over-expression in COS7 cells. Ezrin is important in remodeling cortical actin structures and the formation of membrane filopodia in a manner dependent of Rho GTPases(55).

The up-regulation of Ezrin and the presence of a FERM domain with C-terminus of KRIT1 suggests that this fragment may be important for re-organizing the actin cytoskeleton. Other proteins up-regulated by CT over-expression corroborate this theory.

DKFZp43, Transgelin 2 (SM22), and *LMHX6* are all proposed actin-binding proteins and are up-regulated by CT over-expression(56). Members of the actin protein family, γ-actin and β-actin are also up-regulated by CT over-expression. Yet another actin-related protein up-regulated by CT over-expression is Actinin α 1, an actin cross-linker which modulates actin-based structures such as focal adhesions and membrane ruffles(57). The sheer number of actin-related genes up-regulated by CT over-expression suggest that this fragment of KRIT1 is involved in the re-structuring of the actin cytoskeleton. Although these experiments require confirmation, the results are promising insights into the function of the KRIT1 protein.

Based on our experiments and those of other investigators, a clearer picture of KRIT1 function is emerging. Importantly, our microarray experiments intimate that KRIT1 may not be a simple molecule that binds microtubules and ICAP 1α but one that may help to co-ordinate the cellular cytoskeleton in response to integrin engagement. In response to β-integrin activation, a wide variety of cellular proteins including Rho GTPases, actin-binding proteins and other proteins are activated to produce cellular adhesion structures such as focal adhesions and other important structures involved in cellular migration(58). These coordinated actions in response to integrin engagement are thought to be important for normal vascular development, and it is possible that a full explanation of cavernous malformations fits into these signaling pathways(59).

It is important to acknowledge that our findings were made by using in-vitro cell culture experiments. Cell culture is non-physiologic and results gleaned under such conditions may be the consequence of condition artifacts. However, we believe that our results represent a reasonable start to future investigations into the molecular

pathogenesis of cavernous malformations. Proposed experiments for our laboratory include verification of our initial microarray results by amplifying our microarray genes using DNA from cavernous malformation tissue samples.

CONCLUSIONS

The work in this thesis provides information about *in vitro* cell culture experiments directed at elucidating the function of the KRIT1 gene. The data presented within this body of work expands the previous scope of KRIT1 action and suggests that KRIT1 regulation of the cellular cytoskeleton may include the actin-based structures in addition to previously shown KRIT1 association with microtubules. Localization experiments in spreading endothelial cells show co-localization of KRIT1 and Rac1 a protein known to be involved in widespread rearrangement of the actin cytoskeleton. Rac1 is thought to oppose the formation of focal adhesions, a type of integrin-dependent adhesion process. Importantly, ICAP 1α, one of two known KRIT1-binding proteins, has also been shown to oppose the formation of focal adhesions. These findings suggest that KRIT1, ICAP 1α, and Rac1 may be part of a coordinated integrin signaling mechanisms.

Transfection of KRIT1-GFP constructs lead to interesting cellular phenotypes suggesting active rearrangement of the cellular cytoskeleton. Follow-up experiments with DNA microarrays corroborate the observations made by microscopy on transfected cells. In particular, C-terminal KRIT1-GFP over-expression leads to the up-regulation of several actin-related genes, suggesting that this fragment, with its FERM domain, may regulate actin microfilaments. Follow-up experiments must be done to confirm the results observed in this thesis.

Bibliography

1. Jaffar JJ, A.I., Rosenwasser RH. 1999. Vascular malformations of the central nervous system. Philadelphia: Lippincott Williams & Wilkins. 540.
2. Fields WS, L.N. 1989. *A History of Stroke.* New York, NY: Oxford University Press.
3. Galen. 1962. *On Anatomical Procedures, The Later Books.* Cambridge England: Cambridge University Press.
4. Ball, J. 1910. *Andreas Vesalius, the Reformer of Anatomy.* St. Louis: St Louis: Medical Science Press.
5. Awad, I.A., Robinson, J.R., Jr., Mohanty, S., and Estes, M.L. 1993. Mixed vascular malformations of the brain: clinical and pathogenetic considerations. *Neurosurgery* 33:179-188; discussion 188.
6. Smith, J.L., and Garg, B. 2002. Treatment of arteriovenous malformations of the brain. *Curr Neurol Neurosci Rep* 2:44-49.
7. Detweiler, P.W., Porter, R.W., and Spetzler, R.F. 1999. Spinal arteriovenous malformations. *Neurosurg Clin N Am* 10:89-100.
8. Wilkins, R.H. 1985. Natural history of intracranial vascular malformations: a review. *Neurosurgery* 16:421-430.
9. Amin-Hanjani, S., Robertson, R., Arginteanu, M.S., and Scott, R.M. 1998. Familial intracranial arteriovenous malformations. Case report and review of the literature. *Pediatr Neurosurg* 29:208-213.

10. Willinsky, R.A., Lasjaunias, P., Terbrugge, K., and Burrows, P. 1990. Multiple cerebral arteriovenous malformations (AVMs). Review of our experience from 203 patients with cerebral vascular lesions. *Neuroradiology* 32:207-210.

11. Begbie, M.E., Wallace, G.M., and Shovlin, C.L. 2003. Hereditary haemorrhagic telangiectasia (Osler-Weber-Rendu syndrome): a view from the 21st century. *Postgrad Med J* 79:18-24.

12. Jain, I.S., Sharma, S.K., Dhir, S.P., and Kaul, R.L. 1980. Wyburn-Mason syndrome (a case report). *Indian J Ophthalmol* 28:161-163.

13. Del Curling, O., Jr., Kelly, D.L., Jr., Elster, A.D., and Craven, T.E. 1991. An analysis of the natural history of cavernous angiomas. *J Neurosurg* 75:702-708.

14. Hayman, L.A., Evans, R.A., Ferrell, R.E., Fahr, L.M., Ostrow, P., and Riccardi, V.M. 1982. Familial cavernous angiomas: natural history and genetic study over a 5-year period. *Am J Med Genet* 11:147-160.

15. Russell, D.R., LJ. 1989. *Pathology of Tumors of the Nervous System.* Baltimore: Williams and Wilkins.

16. Clatterbuck, R.E., Eberhart, C.G., Crain, B.J., and Rigamonti, D. 2001. Ultrastructural and immunocytochemical evidence that an incompetent blood-brain barrier is related to the pathophysiology of cavernous malformations. *J Neurol Neurosurg Psychiatry* 71:188-192.

17. Ojemann RG, H.R., Rowell RM. 1988. *Surgical Management of Cerebrovascular Diseases.* Baltimore: Williams and Wilkins.

18. Craig, H.D., Gunel, M., Cepeda, O., Johnson, E.W., Ptacek, L., Steinberg, G.K., Ogilvy, C.S., Berg, M.J., Crawford, S.C., Scott, R.M., et al. 1998. Multilocus

linkage identifies two new loci for a mendelian form of stroke, cerebral cavernous malformation, at 7p15-13 and 3q25.2-27. *Human Molecular Genetics* 7:1851-1858.

19. Heutink, P., Haitjema, T., Breedveld, G.J., Janssen, B., Sandkuijl, L.A., Bontekoe, C.J., Westerman, C.J., and Oostra, B.A. 1994. Linkage of hereditary haemorrhagic telangiectasia to chromosome 9q34 and evidence for locus heterogeneity. *J Med Genet* 31:933-936.

20. McDonald, M.T., Papenberg, K.A., Ghosh, S., Glatfelter, A.A., Biesecker, B.B., Helmbold, E.A., Markel, D.S., Zolotor, A., McKinnon, W.C., Vanderstoep, J.L., et al. 1994. A disease locus for hereditary haemorrhagic telangiectasia maps to chromosome 9q33-34. *Nat Genet* 6:197-204.

21. Shovlin, C.L., Hughes, J.M., Tuddenham, E.G., Temperley, I., Perembelon, Y.F., Scott, J., Seidman, C.E., and Seidman, J.G. 1994. A gene for hereditary haemorrhagic telangiectasia maps to chromosome 9q3. *Nat Genet* 6:205-209.

22. McAllister, K.A., Grogg, K.M., Johnson, D.W., Gallione, C.J., Baldwin, M.A., Jackson, C.E., Helmbold, E.A., Markel, D.S., McKinnon, W.C., Murrell, J., et al. 1994. Endoglin, a TGF-beta binding protein of endothelial cells, is the gene for hereditary haemorrhagic telangiectasia type 1. *Nat Genet* 8:345-351.

23. Shovlin, C.L., Hughes, J.M., Scott, J., Seidman, C.E., and Seidman, J.G. 1997. Characterization of endoglin and identification of novel mutations in hereditary hemorrhagic telangiectasia. *Am J Hum Genet* 61:68-79.

24. Johnson, D.W., Berg, J.N., Gallione, C.J., McAllister, K.A., Warner, J.P., Helmbold, E.A., Markel, D.S., Jackson, C.E., Porteous, M.E., and Marchuk, D.A.

1995. A second locus for hereditary hemorrhagic telangiectasia maps to chromosome 12. *Genome Res* 5:21-28.

25. Berg, J.N., Guttmacher, A.E., Marchuk, D.A., and Porteous, M.E. 1996. Clinical heterogeneity in hereditary haemorrhagic telangiectasia: are pulmonary arteriovenous malformations more common in families linked to endoglin? *J Med Genet* 33:256-257.

26. Johnson, D.W., Berg, J.N., Baldwin, M.A., Gallione, C.J., Marondel, I., Yoon, S.J., Stenzel, T.T., Speer, M., Pericak-Vance, M.A., Diamond, A., et al. 1996. Mutations in the activin receptor-like kinase 1 gene in hereditary haemorrhagic telangiectasia type 2. *Nat Genet* 13:189-195.

27. Calvert, J.T., Riney, T.J., Kontos, C.D., Cha, E.H., Prieto, V.G., Shea, C.R., Berg, J.N., Nevin, N.C., Simpson, S.A., Pasyk, K.A., et al. 1999. Allelic and locus heterogeneity in inherited venous malformations. *Hum Mol Genet* 8:1279-1289.

28. Vikkula, M., Boon, L.M., Carraway, K.L., 3rd, Calvert, J.T., Diamonti, A.J., Goumnerov, B., Pasyk, K.A., Marchuk, D.A., Warman, M.L., Cantley, L.C., et al. 1996. Vascular dysmorphogenesis caused by an activating mutation in the receptor tyrosine kinase TIE2. *Cell* 87:1181-1190.

29. Gunel, M., Awad, I.A., Finberg, K., Anson, J.A., Steinberg, G.K., Batjer, H.H., Kopitnik, T.A., Morrison, L., Giannotta, S.L., Nelson-Williams, C., et al. 1996. A founder mutation as a cause of cerebral cavernous malformation in Hispanic Americans. *New England Journal of Medicine* 334:946-951.

30. Gunel, M., Awad, I.A., Finberg, K., Steinberg, G.K., Craig, H.D., Cepeda, O., Nelson-Williams, C., and Lifton, R.P. 1996. Genetic heterogeneity of inherited cerebral cavernous malformation. *Neurosurgery* 38:1265-1271.

31. Gunel, M., Awad, I.A., Anson, J., and Lifton, R.P. 1995. Mapping a gene causing cerebral cavernous malformation to 7q11.2-q21. *Proceedings of the National Academy of Sciences of the United States of America* 92:6620-6624.

32. Sahoo, T., Johnson, E.W., Thomas, J.W., Kuehl, P.M., Jones, T.L., Dokken, C.G., Touchman, J.W., Gallione, C.J., Lee-Lin, S.Q., Kosofsky, B., et al. 1999. Mutations in the gene encoding KRIT1, a Krev-1/rap1a binding protein, cause cerebral cavernous malformations (CCM1). *Hum Mol Genet* 8:2325-2333.

33. Liquori, C.L., Berg, M.J., Siegel, A.M., Huang, E., Zawistowski, J.S., Stoffer, T., Verlaan, D., Balogun, F., Hughes, L., Leedom, T.P., et al. 2003. Mutations in a gene encoding a novel protein containing a phosphotyrosine-binding domain cause type 2 cerebral cavernous malformations. *Am J Hum Genet* 73:1459-1464.

34. Gunel, M., Laurans, M.S., Shin, D., DiLuna, M.L., Voorhees, J., Choate, K., Nelson-Williams, C., and Lifton, R.P. 2002. KRIT1, a gene mutated in cerebral cavernous malformation, encodes a microtubule-associated protein. *Proceedings of the National Academy of Sciences of the United States of America* 99:10677-10682.

35. Whitehead, K.J., Plummer, N.W., Adams, J.A., Marchuk, D.A., and Li, D.Y. 2004. Ccm1 is required for arterial morphogenesis: implications for the etiology of human cavernous malformations. *Development* 131:1437-1448.

36. Guzeloglu-Kayisli, O., Amankulor, N.M., Voorhees, J., Luleci, G., Lifton, R.P., and Gunel, M. 2004. KRIT1/Cerebral Cavernous Malformation 1 Protein Localizes to Vascular Endothelium, Astrocytes, and Pyramidal Cells of the Adult Human Cerebral Cortex. *Neurosurgery* 54:943-949.

37. Serebriiskii, I., Estojak, J., Sonoda, G., Testa, J.R., and Golemis, E.A. 1997. Association of Krev-1/rap1a with Krit1, a novel ankyrin repeat-containing protein encoded by a gene mapping to 7q21-22. *Oncogene* 15:1043-1049.

38. Bos, J.L., Franke, B., M'Rabet, L., Reedquist, K., and Zwartkruis, F. 1997. In search of a function for the Ras-like GTPase Rap1. *FEBS Lett* 410:59-62.

39. Chang, D.D., Wong, C., Smith, H., and Liu, J. 1997. ICAP-1, a novel beta1 integrin cytoplasmic domain-associated protein, binds to a conserved and functionally important NPXY sequence motif of beta1 integrin. *J Cell Biol* 138:1149-1157.

40. Zhang, J., Clatterbuck, R.E., Rigamonti, D., Chang, D.D., and Dietz, H.C. 2001. Interaction between krit1 and icap1alpha infers perturbation of integrin beta1-mediated angiogenesis in the pathogenesis of cerebral cavernous malformation. *Hum Mol Genet* 10:2953-2960.

41. Zawistowski, J.S., Serebriiskii, I.G., Lee, M.F., Golemis, E.A., and Marchuk, D.A. 2002. KRIT1 association with the integrin-binding protein ICAP-1: a new direction in the elucidation of cerebral cavernous malformations (CCM1) pathogenesis. *Hum Mol Genet* 11:389-396.

42. Berrier, A.L., Mastrangelo, A.M., Downward, J., Ginsberg, M., and LaFlamme, S.E. 2000. Activated R-ras, Rac1, PI 3-kinase and PKCepsilon can each restore

cell spreading inhibited by isolated integrin beta1 cytoplasmic domains. *J Cell Biol* 151:1549-1560.

43. Marchuk, D.A., Srinivasan, S., Squire, T.L., and Zawistowski, J.S. 2003. Vascular morphogenesis: tales of two syndromes. *Hum Mol Genet* 12 Spec No 1:R97-112.

44. Kaverina, I., Krylyshkina, O., and Small, J.V. 2002. Regulation of substrate adhesion dynamics during cell motility. *Int J Biochem Cell Biol* 34:746-761.

45. Aspenstrom, P., Fransson, A., and Saras, J. 2004. Rho GTPases have diverse effects on the organization of the actin filament system. *Biochem J* 377:327-337.

46. Mizuki, N., Kimura, M., Ohno, S., Miyata, S., Sato, M., Ando, H., Ishihara, M., Goto, K., Watanabe, S., Yamazaki, M., et al. 1996. Isolation of cDNA and genomic clones of a human Ras-related GTP-binding protein gene and its chromosomal localization to the long arm of chromosome 7, 7q36. *Genomics* 34:114-118.

47. Saucedo, L.J., Gao, X., Chiarelli, D.A., Li, L., Pan, D., and Edgar, B.A. 2003. Rheb promotes cell growth as a component of the insulin/TOR signalling network. *Nat Cell Biol* 5:566-571.

48. Stocker, H., Radimerski, T., Schindelholz, B., Wittwer, F., Belawat, P., Daram, P., Breuer, S., Thomas, G., and Hafen, E. 2003. Rheb is an essential regulator of S6K in controlling cell growth in Drosophila. *Nat Cell Biol* 5:559-565.

49. Castro, A.F., Rebhun, J.F., Clark, G.J., and Quilliam, L.A. 2003. Rheb binds tuberous sclerosis complex 2 (TSC2) and promotes S6 kinase activation in a rapamycin- and farnesylation-dependent manner. *J Biol Chem* 278:32493-32496.

50. Miyashita, K., Itoh, H., Sawada, N., Fukunaga, Y., Sone, M., Yamahara, K., Yurugi-Kobayashi, T., Park, K., and Nakao, K. 2003. Adrenomedullin provokes endothelial Akt activation and promotes vascular regeneration both in vitro and in vivo. *FEBS Lett* 544:86-92.

51. Kim, W., Moon, S.O., Sung, M.J., Kim, S.H., Lee, S., So, J.N., and Park, S.K. 2003. Angiogenic role of adrenomedullin through activation of Akt, mitogen-activated protein kinase, and focal adhesion kinase in endothelial cells. *Faseb J* 17:1937-1939.

52. Arber, S., and Caroni, P. 1995. Thrombospondin-4, an extracellular matrix protein expressed in the developing and adult nervous system promotes neurite outgrowth. *J Cell Biol* 131:1083-1094.

53. Sun, C.X., Robb, V.A., and Gutmann, D.H. 2002. Protein 4.1 tumor suppressors: getting a FERM grip on growth regulation. *J Cell Sci* 115:3991-4000.

54. Sun, C.X., Haipek, C., Scoles, D.R., Pulst, S.M., Giovannini, M., Komada, M., and Gutmann, D.H. 2002. Functional analysis of the relationship between the neurofibromatosis 2 tumor suppressor and its binding partner, hepatocyte growth factor-regulated tyrosine kinase substrate. *Hum Mol Genet* 11:3167-3178.

55. Nakamura, N., Oshiro, N., Fukata, Y., Amano, M., Fukata, M., Kuroda, S., Matsuura, Y., Leung, T., Lim, L., and Kaibuchi, K. 2000. Phosphorylation of ERM proteins at filopodia induced by Cdc42. *Genes Cells* 5:571-581.

56. Mack, C.P., Somlyo, A.V., Hautmann, M., Somlyo, A.P., and Owens, G.K. 2001. Smooth muscle differentiation marker gene expression is regulated by RhoA-mediated actin polymerization. *J Biol Chem* 276:341-347.

57. Greenwood, J.A., Theibert, A.B., Prestwich, G.D., and Murphy-Ullrich, J.E. 2000. Restructuring of focal adhesion plaques by PI 3-kinase. Regulation by PtdIns (3,4,5)-p(3) binding to alpha-actinin. *J Cell Biol* 150:627-642.

58. Schoenwaelder, S.M., and Burridge, K. 1999. Bidirectional signaling between the cytoskeleton and integrins. *Curr Opin Cell Biol* 11:274-286.

59. Ruegg, C., and Mariotti, A. 2003. Vascular integrins: pleiotropic adhesion and signaling molecules in vascular homeostasis and angiogenesis. *Cell Mol Life Sci* 60:1135-1157.

HARVEY CUSHING/JOHN HAY WHITNEY MEDICAL LIBRARY

MANUSCRIPT THESES

Unpublished theses submitted for the Master's and Doctor's degrees and deposited in the Medical Library are to be used only with due regard to the rights of the authors. Bibliographical references may be noted, but passages must not be copied without permission of the authors, and without proper credit being given in subsequent written or published work.

This thesis by
has been used by the following person, whose signatures attest their acceptance of the above restrictions.

NAME AND ADDRESS **DATE**

YALE MEDICAL LIBRARY
3 9002 01065 6354